7.

The Effects of Steroids on the Skeleton of the Poecillid Fish *Lebistes reticulatus.*

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(Plate I).

The effect of the sex hormones and extracts of the anterior lobe of the pituitary upon the structure of bone has been studied by various investigators. Estrogen-treated mice, guinea pigs, pigeons and chickens have shown, upon histological study of the skeleton, an increased ossification, at least in certain regions. In order to help complete the vertebrate phylogenetic picture, the work reported here was designed to extend this study to fish. The effects of pregneninolone (Pranone, ethinyl testosterone, anhydrodroxy-progesterone) and alpha estradiol (Progynon DH) upon the skeletal structures of *Lebistes reticulatus* (Peters), a fish in which the secondary sex characters are highly responsive to these substances, were determined and are reported here. In this species, the effects of pregneninolone are purely androgenic, but in mammals this steroid substance has various other actions.

The author wishes to express his appreciation to Drs. Robert Gaunt and C. M. Breder, Jr., for suggestions and assistance. He is also indebted to Dr. Max Gilbert of the Schering Corporation for supplying the hormones used in this work. The data presented here are taken in part from a thesis for the degree of Master of Science at New York University.

Ten to fifteen fish were kept in two and a half gallon aquaria. Several plants were grown in each aquarium in an inch of sand. The temperature of the water was controlled thermostatically and ranged from 74°-78° F. Each tank was fitted with a porous stone through which was pumped a steady stream of air. Both treated and control fish were fed, daily, a diet of dried tropical fish food which was supplemented by Daphnia and Tubifex once a week.

Lebistes reticulatus, a viviparous poecillid with marked sexually dimorphic characteristics, was used as the experimental animal. In this species the anal fin of the adult male is modified to form a gonopodium, the body size of the male is normally smaller than that of the female, and the male only is brilliantly colored. The reaction of these sexually dimorphic traits of *Lebistes* to the two steroids used here have been previously described by Berkowitz (1) and Eversole (6). Since their results were fully confirmed in the course of our observations no duplicate description is given of them.

A total of 67 fish were treated. Thirty received estradiol, contained in tablets made for human oral use which were powdered and sprinkled on the water. Total dosage varied from 6.5 to 15 milligrams per tank given in equal weekly portions for periods varying from 18 to 110 days. A similar procedure was followed in treating fish with 5-milligram doses of pregneninolone, except that the effects of a single treatment were so long-lasting that monthly administration was more than sufficient to maintain masculinization of all secondary sex characters. In fact, to eliminate effective amounts of the steroid from the tanks a very thorough cleaning had to be done even six months after a dose of the compound was added to the tanks.

Histological studies were made on a segment cut transversely just caudal to the pectoral fin and between the posterior end of the body cavity and the caudal fin. The whole skeleton was studied in total mounts. The material was fixed in Bouin's fluid, washed in 70% alcohol, dehydrated by the dioxan method, and embedded in tissue-mat. Serial sections were cut at 10 microns in thickness and were stained either with Harris' hematoxylin and counterstained with eosin, or with Mallory's triple stain. Whole mounts were prepared by clearing freshly killed fish in KOH until the muscular and skeletal portions were translucent and then staining with Alizarin dye as described by Hollister (10).

Since the treatments given either masculinized or feminized the external sexual characteristics of the animals, the original sex was determined at the end of the experiment by histological examination of the gonads. These were routinely sectioned in the course of other observations. The gonad is itself affected by these treatments (1, 6),

but whether it was originally a testis or ovary was generally easily discernible by its histological appearance, the essential gametogenic elements of which were not changed. The sex ratio of the strain of fish used here is 1:1.

Observations.

I. Sexual dimorphism in the skeleton.

Cleared whole mounts of normal male and female *Lebistes* showed a skeletal dimorphic character, aside from the difference in size, only in the region of the anal fin or gonopodium (male). The skeleton which supports the anal fin of the adult female (Fig. 2) and of immature fish is composed of a group of ten separate interhaemal bones which project dorsally from the anal fin toward the haemal spines of the first three caudal vertebrae.

Three of the interhaemal bones, the second, third and fourth, are fused as one bone in the adult male (Fig. 1). In addition the first three caudal vertebrae are markedly enlarged and make a sharp angle in the direction of the three fused interhaemal bones.

Most bones in this species are made up of a central cartilaginous mass surrounded by a crust-like ossified layer. No sexual dimorphism in the histology of these structures was noted, except again in the anal fin region where the interhaemal bones of the male contained much thicker ossified layers.

II. The effects of hormones upon the skeleton.

All fish, regardless of sex, fed 5 milligrams of pregneninolone at birth, developed typical male structures precociously in the anal fin region described above. There was a fusion of four and sometimes five of the interhaemal bones in the treated animals (Figs. 3, 4, 7). This steroid caused a dwarfed condition in *Lebistes* and consequently the whole skeletal apparatus of the treated fish was reduced in size (Fig. 3). Adult females, given a single feeding of 5 milligrams of pregneninolone, all developed interhaemal bones much like the adult males (Fig. 4) within three weeks. Normal males show this skeletal dimorphism 50-60 days after birth.

Fifteen fish were fed alpha estradiol from birth. All developed a typical female anal fin with the 10 separate interhaemal bones attached (Figs. 2, 6). One group of 9 fish received 3.25 milligrams of the hormone over a period of 50 days. The male controls had begun to show a difference in the anal fin skeleton but not the treated animals. Continuing treatment until 110 days in another series did not vary results.

All of the estrogen-treated fish were studied for the effect upon ossification of the interhaemal bones. It was found in every case that there was no increase in ossification and these bones had the exact appearance of the female untreated animals (Figs. 5, 6).

On the other hand, all of the fish that were fed pregneninolone, without exception, showed an increase deposition of bone around the central cartilaginous structure of the interhaemal bones toward the condition of the adult male (Fig. 7). The first three haemal spines showed an increase in size which approached that of a normal adult male.

The supporting structures of the gonadopodium itself undergo profound changes in the course of sexual maturation in the male. These are induced precociously by pregneninolone, as described by Eversole (6).

The vertebral column was studied carefully for possible effects of hormone treatment. In all of the 67 fish treated with either hormone there was no increase in ossification or other histological changes in the vertebrae, except for the larger spines associated with the anal fin apparatus of the pregneninolone-treated fish.

Discussion.

The failure of estrogenic substances in the amounts used here to produce any effect upon ossification is conflicting with the results obtained with this hormone in higher forms. Gardner and his co-workers have reported an increase in endosteal bone formation in birds (12, 14, 15) and mice (7, 8, 24) and some increase in periosteal bone in the pigeon (25). Sutro (21) reported that there was no change in periosteal bone in estrogen-treated mice, although increases in new bone formation of the medullary cavity were noted. The changes in bone formation in rats seemed to be confined to the area round the epiphysis of the long bones, where an increase in the density of this area occurred (Day and Follis, 11). Silberberg and Silberberg (19, 20) found that both estrogens and androgens intensified the ossification of cartilage and increased the deposition of bone in the guinea pig, but that the effects of the androgens were much less intense. Perhaps an explanation for the lack of increased ossification of the estrogen-treated fish is the absence of true endosteal bone and the presence primarily of membrane bone which has been described for certain parts by Moorkerjee (16). The literature seems to reveal no information at present as to the effects of estrogens upon the membrane bones of higher vertebrates.

Pregneninolone with its androgenic action in *Lebistes*, stimulates ossification in certain of the bones of this fish. This is just the opposite of the effects produced by testosterone in birds and most mammals.

Gardner and Pfeiffer (9) and Turner and co-workers (23) found that testosterone propionate inhibited hypercalcification in the mouse and rat. In ducks, Landauer (15) reported that estrogens caused hyper-ossification even in the presence of testosterone. In view of the difference in size of the two sexes of the guppy, the female being the larger, it would be logical to expect that if sex steroids had any effect on ossification, the androgens would stimulate the process thus stopping growth. However, the well established inhibition of growth in these fish by androgens cannot be attributed to a hyper-ossification, since the latter occurs only in the region of the anal fin.

Finally, it should be pointed out that the regions of the skeleton affected by hormone treatment are exclusively those in which a normal sexual dimorphism exists. The changes induced were in character and extent parallel to that expected from the picture of normal sexual dimorphism. In other words, this fish seems to be refractory to overdosage effects and most parts of its skeleton are probably totally unresponsive to sex steroids, except in generalized size difference.

The variability and specialization in teleost fish is such that experimental results of the type reported here may be applicable only to the species in which they are observed.

Summary.

1. Most parts of the skeleton of *Lebistes reticulatus* are not responsive to either estrogenic (alpha estradiol) or androgenic (pregneninolone) sex steroids, except in size. Pregneninolone caused a reduction in skeletal size, associated with a generalized dwarfing, while alpha estradiol produced no clearly significant change in size.

2. Skeletal constituents associated with the anal fin (the gonopod in the male) differ markedly in the two sexes. This anal fin of the immature fish of both sexes is supported by a group of ten separate interhaemal bones. These bones are entirely cartilage in the young fish and in the adult female they are surrounded by a thin layer of membrane bone. In the adult male, these bones are made up of thicker portions of membrane bone than in the female and, in addition, the second, third, and fourth interhaemal bones are fused as one. The male-like condition can be fully developed by androgens in either sex and the female condition can be induced by estrogens.

No changes in the anal fin region could be induced which were greater in extent than those normally seen—that is, there were no apparent overdosage phenomena under the conditions of these experiments.

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EXPLANATION OF THE PLATE.

PLATE I.

Figs. 1 - 4, inclusive, are camera lucida drawings made from whole mounts which had been stained with Alizarin dye. They are all of the same magnification.

Fig. 1. Lateral view of the skeleton of an adult male, in the mid-body region, showing three enlarged haemal spines and the fusion of three of the interhaemal bones. These characters are typical of the mature male.

Fig. 2. Lateral view of the skeleton of an adult female, in the mid-body region, showing small normal haemal spines and ten separate interhaemal bones, typical of the adult female and the immature fish.

Fig. 3. Lateral view of the skeleton, in the mid-body region, of a female guppy fed 5 milligrams of pregneninolone from birth for a period of 21 days showing the enlarged haemal spines of the first three caudal vertebrae and the fusion of 5 interhaemal bones. These characters are typical of the adult male.

Fig. 4. Lateral view of the skeleton, in the mid-body region, of an adult female, treated with 5 milligrams of pregneninolone over a period of 21 days, showing slightly enlarged haemal spines of the first three caudal vertebrae and the fusion of the first 4 interhaemal bones. These are typical male characters.

Fig. 5. A median sagittal section of the mid-body region of an adult female 110 days old, showing separate interhaemal bones with a central cartilage portion which is surrounded by a thin sheath of membrane bone. × 100.

Fig. 6. A median sagittal section of the mid-body region of a 110-day-old male treated with 5 milligrams of estradiol from birth. The structure of the separate interhaemal bones is similar to the female control (Fig. 5). × 100.

Fig. 7. A median sagittal section of the mid-body region of a female animal treated from birth with 5 milligrams of pregneninolone over a period 21 days showing fused interhaemal bones and a thick layer of membrane bone surrounding the central portion of cartilage. × 100.

ABBREVIATIONS.

H S	Haemal spine.
F I S	Fused interhaemal bone.
A N T	Anterior end of skeleton.
R	Radial bulbous portion of the anal fin to which are attached the fin rays.
I S	Interhaemal bone or spine.
C	Cartilage.
B	Membrane bone.

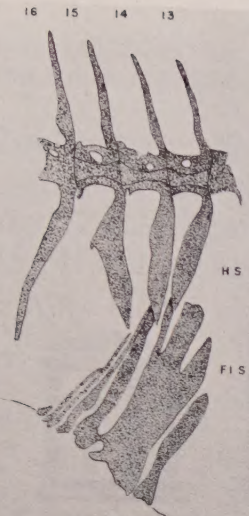


FIG. 1.

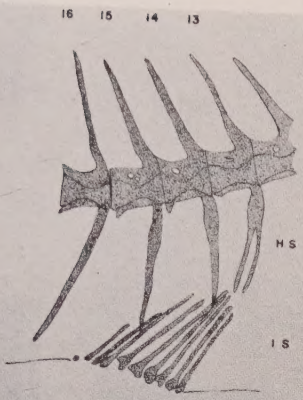
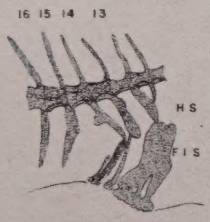


FIG. 2.



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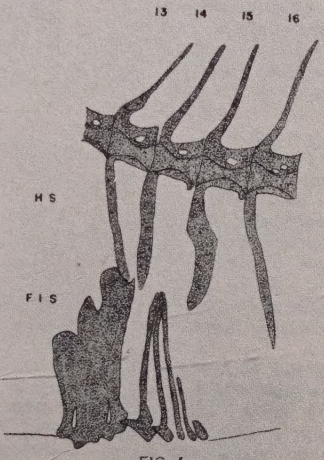

FIG. 4.

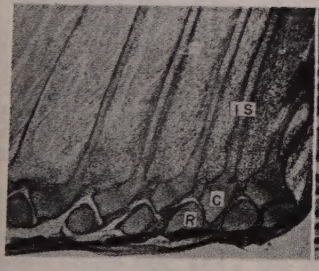

FIG. 5.

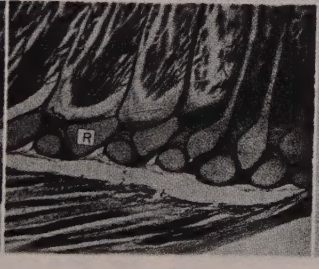

FIG. 6.

FIG. 7.

THE EFFECTS OF STEROIDS ON THE SKELETON OF LEBISTES RETICULATUS.